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Capsids Are Formed in a Mutant Virus Blocked at the Maturation Site of the UL26 and UL26.5 Open Reading Frames of Herpes Simplex Virus Type 1 but Are Not Formed in a Null Mutant of UL38 (VP19C)

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Previously we reported that null mutant viruses of UL19 (VP5) or of UL18 (VP23), essential components of herpes simplex virus type 1 (HSV-1) capsid shells, do not form precursor capsid structures as judged by sedimentation and electron microscope analysis. A goal of the present experiments was to isolate a null mutant virus for the remaining essential component of capsid shells, VP19C, encoded by the UL38 open reading frame (ORF). Furthermore, we wished to determine if a virus altered in the UL26 maturation cleavage site at residues 610 and 611 produced a lethal phenotype. Therefore, we decided to isolate cell lines that encode and express multiple capsid genes. Several cell lines were isolated by transformation of Vero cells and one designated C32 expressed all of the essential capsid proteins. Using this cell line we isolated a null mutant virus in the UL38 ORF and a mutant virus that was altered at residues 610 and 611 of the UL26 and UL26.5 gene products. We found that the null mutant in VP19C did not form a detectable product as judged by sedimentation and electron microscope analyses following infection of nonpermissive cells. The mutant virus altered at the UL26 maturation site resulted in the accumulation of B capsids. Therefore, cleavage at this site was essential for the maturation of B capsids into C capsids. Interestingly, the absence of cleavage at the maturation site was required for the retention of VP24 in the capsid.

INTRODUCTION

The herpes simplex virus type 1 (HSV-1) B capsid is composed predominantly of seven capsid proteins. Their designation, and the open reading frames (ORF) encoding the proteins, are VP5 (UL19), VP19C (UL38), 21 (UL26), 22a (UL26.5), VP23 (UL18), VP24 (UL26), and VP26 (UL35) (Gibson and Roizman, 1972; Heilman *et al.,* 1979; Cohen *et al.,* 1980; McGeoch *et al.,* 1988). VP5, the major capsid protein, in association with scaffold molecules (the more abundant 22a and probably the less abundant 21), forms the 150 hexons and 12 pentons present in capsid shells (Newcomb *et al.,* 1989, 1993; Booy *et al.,* 1991; Zhou *et al.,* 1994). Adjacent pentons and hexons are held together, at least in part, by a complex of VP19C and VP23, referred to as the triplex because of its three-pronged structure in cryoelectron micrographs (Newcomb *et al.,* 1993; Zhou *et al.,* 1994; Trus *et al.,* 1996). VP26 is located at the outer surface of VP5 hexons, but not pentons (Booy *et al.,* 1994; Trus *et al.,* 1995; Zhou *et al.,* 1995). It is not essential for capsid formation in mammalian cell culture (Desai *et al.,* 1998), in insect cells that express all of the capsid proteins except VP26, (Tatman *et al.,* 1994; Thomsen *et al.,* 1994), or in capsids formed *in vitro* from proteins expressed in insect cells (Newcomb *et al.,* 1996). However, the absence of VP26 in the virion results in a reduction in the number of infectious viruses produced during replication in the mouse trigeminal ganglia by 30- to 100-fold (Desai *et al.,* 1998).

Three of the B capsid proteins are encoded by the UL26 and UL26.5 ORFs from two 3′ coterminal transcripts (Holland *et al.,* 1984; Liu and Roizman, 1991a). One transcript, designated UL26, is initiated 180 nucleotides upstream of the entire coding sequence, and the second, designated UL26.5, is initiated near the center of the ORF (1000 nucleotides downstream of the first transcript). The UL26 ORF (635 amino acid residues) encodes a protease (Liu and Roizman, 1991b; Preston *et al.,* 1992) and cleavage occurs at two sites, a maturation site between amino acid residues 610 and 611 and a release site between residues 247 and 248 (Dilanni *et al.,* 1993). The proteolytic activity specified by UL26 has been localized to the N-terminal 247 residues (Liu and Roizman, 1993; Weinheimer *et al.,* 1993). The products of the release site cleavage generate B capsid proteins VP24 and 21 (at the N-terminus and C-terminus, respectively) (Weinheimer *et al.,* 1993). The more abundant 22a is encoded by the UL26.5 transcript and is initiated at codon 307 (Liu and Roizman, 1991a,b). Therefore, 21 and

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22a are identical in amino acid sequence between residues 307 and 635, and both proteins can specify scaffold activity (Tatman *et al.,* 1994; Thomsen *et al.,* 1994; Robertson *et al.,* 1996). Throughout the text UL26 ORF numbers will be used to designate residues in VP24, 21, or 22a. Homologous proteins encoded by human and simian cytomegalovirus (CMV) have been extensively characterized by Gibson and his colleagues (see, for example, Welch *et al.,* 1991 and Gibson *et al.,* 1994).

During the assembly of B capsids, interactions may occur between VP5 monomers as well as between VP5 and each of the remaining capsid proteins. Cellular localization studies using fluorescently tagged antibodies show that VP5 is transported into the nucleus more efficiently in the presence of the precursor form of 22a than in its absence and that VP5 and VP23 are transported efficiently in the presence of VP19C (Nicholson *et al.,* 1994; Rixon *et al.,* 1996). Using the yeast two-hybrid system interactions were detected between VP5 and 22a, a strong self-interaction among 22a proteins, and an interaction between VP19C and VP23. Interactions between VP5 and 22a (encoded by UL26.5) were also observed if the genes encoded by the UL26 ORF were used (Desai and Person, 1996). The same interactions were detected for the homologous proteins of human CMV (Wood *et al.,* 1997; Gibson *et al.,* 1996). A major self-interaction region of 22a included residues 457 to 504 of the UL26 ORF (Desai and Person, 1996), and recently key residues within that region were identified (Pelletier *et al.,* 1997). The 25 amino acids C-terminal to the maturation site specified by UL26 and UL26.5 are necessary for interaction with VP5 (Thomsen *et al.,* 1995; Desai and Person, 1996; Hong *et al.,* 1996). Capsid shells, but not sealed capsids, are formed in the absence of the C-terminal 25 amino acids of the UL26 and UL26.5 ORFs (Kennard *et al.,* 1995; Matusick-Kumar *et al.,* 1995; Thomsen *et al.,* 1995). For HSV-1 a minimal interaction sequence was mapped to residues 622 to 633 using a GST-pulldown assay (Hong *et al.,* 1996). Furthermore, residues 623 to 625 were shown to be sufficient determinants to change the specificity of the reaction from HSV-1 to HCMV (Beaudet-Miller *et al.,* 1996). The interaction between the C-terminus of 22a and VP5 is enhanced by the self-interaction domain of 22a (Nicholson *et al.,* 1994; Pelletier *et al.,* 1997). Although the region of 22a that interacts with VP5 has been extensively studied the region of VP5 that interacts with 22a is not known.

Herpesviruses are unique in their capsid makeup in having adjacent pentamers and hexamers of the major capsid protein held together by a complex of two other capsid and viral proteins, VP19C and VP23. VP19C–VP23 complexes have been observed in lysates of insect cells that express these proteins (Spencer *et al.,* 1998). In their experiments the complex, but not the individual proteins, bound to VP5 in the presence of scaffold molecules to form capsids *in vitro.* The proteins of HCMV that are homologous to VP19C and VP23 have also been detected as a complex by coprecipitation and sedimentation techniques (Baxter and Gibson, 1998).

Several years ago we reported that a null mutation in the gene encoding VP23 did not form capsids upon infection of nonpermissive cells (Desai *et al.,* 1993). Two years ago we began to isolate a null mutant virus in the gene encoding VP19C. At that time it was shown that insect cells infected with baculoviruses that encode all of the capsid proteins form an abundant amount of B capsids (Tatman *et al.,* 1994; Thomsen *et al.,* 1994) and that capsids were not formed if the virus encoding VP19C (UL38) was omitted from the infection. Earlier, Pertuiset *et al.* (1989) showed that a temperature-sensitive mutation in UL38 did not give rise to capsids at the nonpermissive temperature. Therefore it appeared that capsids would not be assembled in a virus that lacked VP19C. Nevertheless, we felt that the above observations needed to be confirmed using a null mutant virus in the UL38 gene. A cell line was also generated that was transformed for all the genes specifying the essential capsid proteins (UL18, UL19, UL26, and UL38). Such a cell line is needed to construct multiple mutants in the genes encoding these essential capsid proteins. This cell line was capable of propagating mutants in the genes encoding VP5, VP23, and UL26 and was used to isolate the VP19C null mutant virus and a virus that was blocked at the maturation cleavage site of UL26 (residues 610/611). The characterization of these mutant viruses is described below.

RESULTS

Isolation of cell lines that express multiple capsid proteins

In order to isolate viruses that specify mutations in more than one essential capsid gene, cell lines are needed that express these genes. Since three relatively small plasmids could be cloned that encode the essential capsid proteins (UL18, UL19, UL26, UL26.5, and UL38) we decided to attempt to isolate a single cell line that expressed all of the essential capsid proteins. Vero cells were cotransformed with the three plasmids (pKUL18-19, pKUL26, and pKUL38) and with pSV2neo at molar ratios of four for each HSV-1 plasmid to one for pSV2neo. Sixty-one neomycin-resistant colonies were isolated and tested for the ability to support the growth of null mutant viruses. Four supported the growth of mutant viruses in UL18 (K23Z) and in UL19 (K5ΔZ) (Desai *et al.,* 1993). Three of these also supported the growth of the null mutant virus in UL26 (KUL26ΔZ) (Desai *et al.,* 1994), and one of the three cell lines, designated C32, supported the growth of the UL38 temperature-sensitive mutant A44ts2 at the nonpermissive temperature (Pertuiset *et al.,* 1989). C32 was used to isolate a null mutant virus in UL38 which encodes VP19C. The yield of mutant

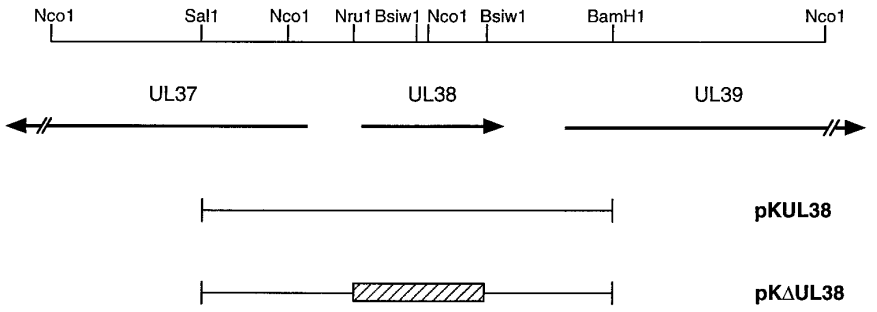

FIG. 1 The UL38 region of the HSV-1 (KOS) genome. Restriction sites relevant to the current study are indicated in the top line. UL37, UL38, and UL39 ORFs (McGeoch *et al.,* 1988) are indicated in the second line. Sequences encoded by pKUL38 and the extent of the deletion in pKΔUL38 are also shown.

viruses in the other essential capsid genes (KΔ5Z, K23Z, and KUL26ΔZ) was determined after a single cycle of growth in C32 cells. Viruses were harvested 24 h after infection at an m.o.i. of 10 PFU/cell using monolayer cultures in six-well trays. The average yield was 1200 PFU/cell for KOS, 535 for KUL26ΔZ, and 455 for KΔ5Z and K23Z.

Isolation and characterization of a virus specifying a null mutation in the gene encoding VP19C

A mutant plasmid with a 1.26-kb deletion which included almost all of the UL38 ORF was constructed from pKUL38 as shown in Fig. 1 pKΔUL38 was cotransfected along with intact KOS DNA into C32 cells in a marker transfer experiment. Infected cells were harvested and titered 48 h later and approximately 30 PFU was added to the same cells in 96-well trays. Wells were identified that contained single plaques and viruses in these wells were screened for growth on C32 and Vero cells. A virus was found that grew on C32 but not on Vero cells. This virus was plaque purified twice and a working stock of virus was prepared on the permissive cells. The virus was designated KΔ19C. Yields of approximately 50 PFU/cell were obtained and the titer on permissive cells was 1×10^4 times that on Vero cells. Since the virus requires a cell line that expresses VP19C for propagation, VP19C is essential for viral growth in cell culture. This virus was used in the studies described below. The low yield of 50 PFU/cell for the newly isolated KΔ19C virus was disappointing and may be related to the fact that we were isolating KΔ19C and the transformed cell lines at the same time.

Southern blot analysis of the mutant virus DNA was performed to determine the genotype of the virus in the UL38 region of the KOS genome. Viral DNA was prepared and cleaved with *Bam*HI and *Sal*I or with *Nco*I, and the restriction fragments were separated by agarose gel electrophoresis. Fragments were blotted onto nitrocellulose and probed with radioactively labeled pKUL38 (Fig. 2). For KOS DNA cleaved with *Bam*HI and *Sal*I the probe hybridized to the 3.9-kb fragment that encodes UL38 (Fig. 2, lane 1). For KΔ19C the probe hybridized to a single fragment shortened by 1.26 kb (lane 2). Digestion of KOS DNA with *Nco*I (see Fig. 1) gave three fragments (3.8, 2.2, and 1.4 kb, see lane 3). For the mutant virus the 2.2-kb fragment from the UL37 region was unaltered by the deletion, while the two remaining fragments were fused and shortened by 1.26 kb (lane 4). This analysis confirmed that the plasmid-specified mutation was introduced into the virus genome. Furthermore, marker rescue experiments were carried out in C32 cells by cotransfecting the cells with KΔ19C viral DNA and either the pKUL38 plasmid or the control plasmid pKNot1 (UL26). The results from these experiments gave a marker rescue frequency of the VP19C mutant of 8.2% with pKUL38 and less than 1.1% with the control (pKNot1) plasmid. This further confirmed the genotype of KΔ19C.

To investigate the synthesis of the VP19C polypeptide, Vero and C32 cells were infected with KΔ19C and KOS viruses at an m.o.i. of 10 PFU/cell. Infected cells were

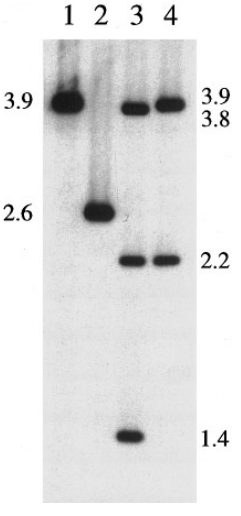

FIG. 2. Southern blot analysis of KΔ19C viral DNA. Viral DNA for KOS (lanes 1 and 3) and for KΔ19C (lanes 2 and 4) was digested with *Bam*HI and *Sal*I (lanes 1 and 2) or with *Nco*I (lanes 3 and 4) and the fragments were resolved by agarose gel electrophoresis. Filters were probed with ^{32}P-labeled pKUL38.

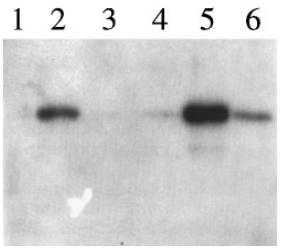

FIG. 3. Western blot analysis of KΔ19C-infected cells. Vero (lanes 1 to 3) and C32 cells (lanes 4 to 6) were infected with KΔ19C (lanes 3 and 6) or KOS (lanes 2 and 5) or mock-infected (lanes 1 and 4) and harvested 24 h postinfection, and infected cell proteins were separated by SDS–PAGE (12% acrylamide). Proteins were electroblotted onto filters and probed for the expression of VP19C using antibody NC2. The amount of lysate loaded in lane 6 was 2.5× that loaded in lane 5. The low reactivity of the antibody to VP19C observed in lane 4 is due to overflow from lane 5.

harvested 24 h after infection and expression of VP19C was assayed by Western blots (Fig. 3) using antibody NC2 prepared to VP19C obtained from B capsids by Drs. R. Eisenberg and G. Cohen. A band of radioactivity of the appropriate size (50 kDa) was observed for nonpermissive and permissive cells infected with wild-type virus (Fig. 3, lanes 2 and 5, respectively) but is absent in mutant-infected Vero cells (lane 3). A band, albeit of less intensity, is also apparent for C32 cells infected with the mutant virus (lane 6).

To determine if any precursor structures are formed in KΔ19C infected nonpermissive cells, nuclear lysates obtained from [^{35}S]methionine-labeled infected Vero cells were layered onto sucrose gradients and sedimented. Fractions were collected and analyzed by SDS–PAGE (Fig. 4). For KOS-infected cells (Fig. 4A), fractions containing C (fraction 12), B (fraction 9), and predominantly A (fraction 7) capsids were apparent. The positions of the seven capsid proteins present in B capsids are shown on the left side of the figure. A and C capsids lack the scaffold proteins 22a and 21 and therefore are composed of five proteins. DNA packaging results in the more rapidly sedimenting C capsids. Since A and B capsids were only partially resolved on these gradients some 22a was present in fraction 7. Results obtained using KΔ19C are shown in Fig. 4B. Neither the fractions corresponding to A, B, or C capsids for KOS nor fractions closer to the top of the gradient show any capsid sedimenting material by this analysis.

Ultrastructural analysis of mutant-infected cells was also performed to determine whether structures visible by the electron microscope could be detected. Vero cells were infected with KΔ19C and the nuclei in thin sections examined by the electron microscope at 16 h postinfection (see Fig. 5A). No structures were detected in any of the sections examined, confirming the data from the sedimentation analysis.

Isolation and characterization of the UL26 maturation cleavage site mutant virus

Since C32 cells expressed UL26 as well as UL38, a virus specifying a mutation in the maturation cleavage site of UL26 could be constructed to determine whether cleavage at this site is essential for virus replication. To construct the UL26 mutant virus we decided to alter the residues at 610 and 611 of the UL26 and UL26.5 genes so that the protease is not able to cleave at this site. Mutations at these sites were introduced into a plasmid that encoded the UL26 ORF using single-stranded DNA oligonucleotide mutagenesis. The Ala–Ser-encoded residues were changed to Glu–Phe codons which also introduced an *Eco*RI site. The mutation was transferred to the KOS genome by homologous recombination. A mutant virus, designated KUL26-610/611, was identified that grew on C32 but not on Vero cells. Following plaque purification a working stock was prepared on C32 cells and samples of the stock were used in the following experiments.

The presence and the location of the new *Eco*RI site in KUL26-610/611 was confirmed by Southern blot analysis (Fig. 6). The virus genome was probed with a 6.5-kb *Not*I fragment that encodes UL26 (Desai *et al.,* 1994). Cleav-

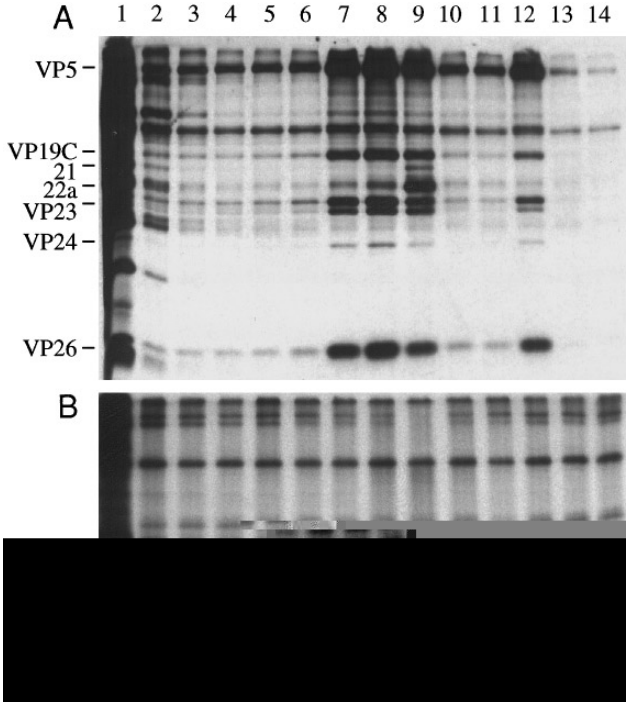

FIG. 4. Sedimentation analysis of nuclear lysates. Vero cells were infected with KOS (A) and KΔ19C (B) at an m.o.i. of 10 PFU/cell and their proteins were labeled from 8 to 24 h postinfection. Nuclear lysates were sedimented through sucrose and the resulting fractions were analyzed by SDS–PAGE (17% acrylamide). The direction of sedimentation was from left to right. Profiles representative of A (fraction 7), B (fraction 9), and C (fraction 12) capsids were seen for KOS, but not for the mutant virus. The positions of B capsid proteins are indicated on the left of A.

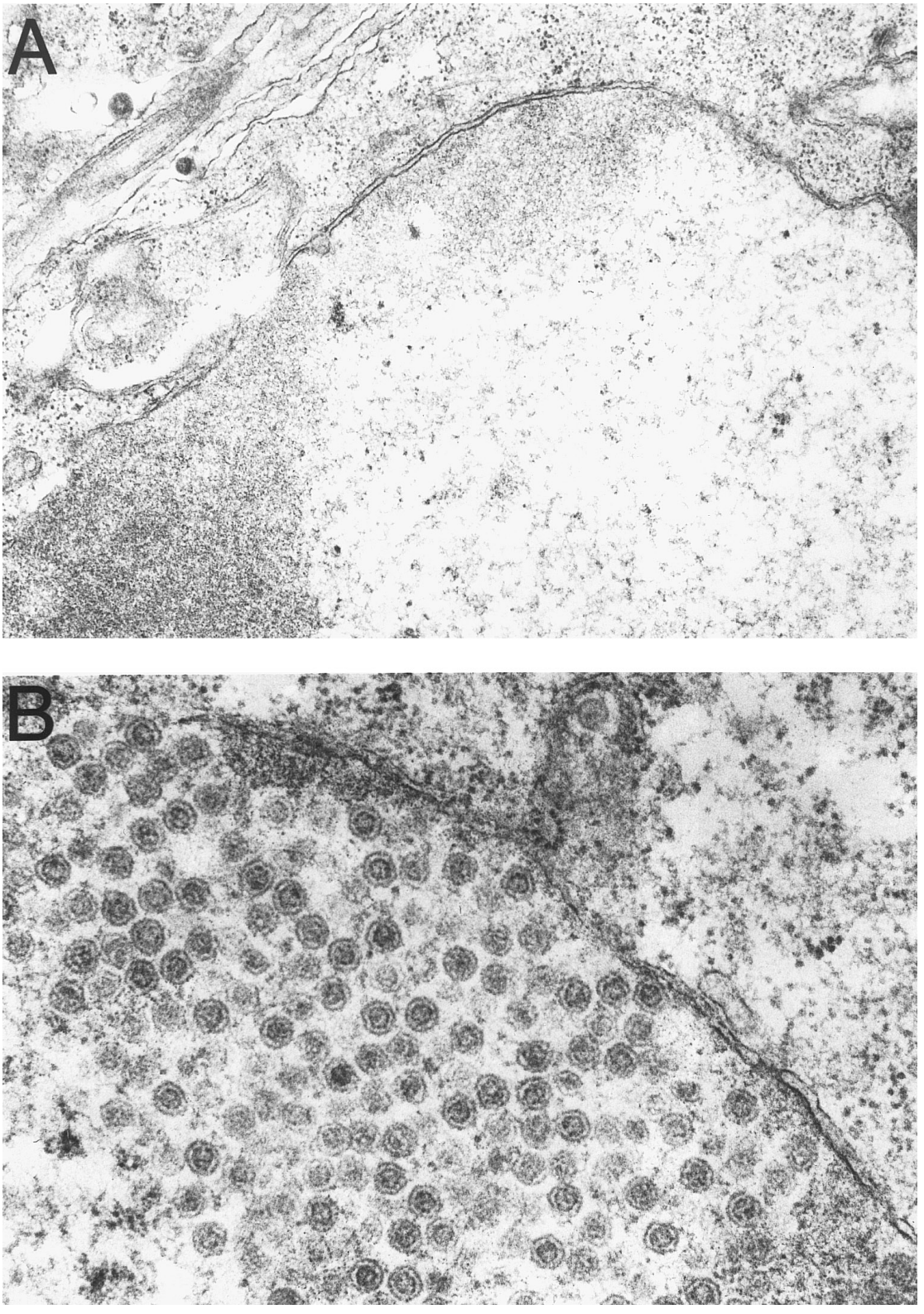

FIG. 5. Electron micrographs of thin sections of infected cell nuclei. Monolayers of Vero cells were infected with KΔ19C (A) and KUL26-610/611 (B) at an m.o.i. of 10 PFU/cell. At 16 h postinfection, cells were fixed and processed for electron microscopy as described under Materials and Methods. Original magnifications were 27,000× for (A) and 54,000× for (B). Electron-microscopy was carried out by Dr. Marco Chacon of Paragon Biotech, Johns Hopkins Bayview Campus, Baltimore.

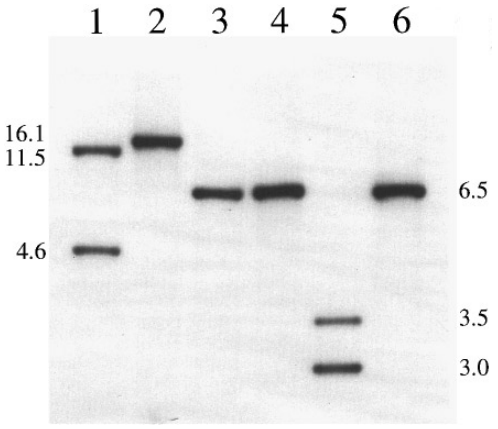

FIG. 6. Southern blot analysis of KUL26-610/611 viral DNA. Viral DNA of mutant (lanes 1, 3, and 5) and wild-type (lanes 2, 4, and 6) viruses was digested with *Eco*RI (lanes 1 and 2), *Not*I (lanes 3 and 4), and with both enzymes (lanes 5 and 6). The fragments were resolved by agarose gel electrophoresis, transferred to filters, and probed with a radioactively labeled *Not*I DNA fragment encoding the UL26 ORF.

age with *Not*I revealed a 6.5-kb band for mutant and KOS DNA (Fig. 6, lanes 3 and 4). Cleavage with *Eco*RI gave the 16.1-kb *Eco*RI wild-type fragment (lane 2) and two fragments of 4.6 and 11.5 kb for the mutant DNA (lane 1). When the two enzymes were used together the 6.5-kb *Not*I fragment was cleaved into 3.5- and 3.0-kb fragments in the case of the mutant virus (lane 5). This analysis confirmed that the plasmid-specified mutation was introduced into the virus genome; hence, the mutation at the maturation site results in a lethal phenotype. Furthermore, marker rescue experiments were carried out in C32 cells by cotransfecting the cells with KUL26-610/611 viral DNA and either the pKNot1 (UL26) plasmid or the control plasmid pKUL38. The results from these experiments gave a marker rescue frequency of the UL26 mutant of 12.7% with pKNot1 and less than 0.5% with the control (pKUL38) plasmid. This further confirmed the genotype of KUL26-610/611.

UL26 mutant viruses that encode an inactive protease (Preston *et al.,* 1992; Thomsen *et al.,* 1995) or a null mutant protease (Gao *et al.,* 1994) assembled capsids that were detected using electron microscopy and sedimentation analysis. To confirm that B capsids were formed following infection of nonpermissive cells, [^{35}S]methionine-labeled nuclear lysates were prepared and analyzed as in Fig. 4. For the mutant virus a single peak of radioactivity was detected in the fractions containing B capsids for wild-type infections. SDS–PAGE analysis of the peak fractions are shown in Fig. 7 for KOS and for KUL26-610/611 (lanes 3 and 4, respectively). The positions of the B capsid proteins from KOS infections are marked at the left of the figure. All of the proteins are present and in similar quantities for capsids formed by the mutant viruses, except VP24. Antibody precipitation of the same sample with MCA406 (lanes 2 and 6), which recognizes an internal region of the scaffold proteins, shows that the proteins (21 and 22a) for KOS (lane 2) are also present for KUL26-610/611 (lane 6) and are shifted to lower mobility positions on the gel, consistent with the absence of protease cleavage at the maturation site. To analyze the relative amounts of precursor (uncleaved) and mature (cleaved) species of scaffold proteins in the capsids an antibody specific to the C-terminal 25 amino acid residues (UL26-C) was used (lanes 1 and 5). Most of the scaffold proteins in B capsids produced during KOS infection were of the mature form since very little radioactivity was detected (lane 1). By contrast a significant amount of radioactivity was detected for capsids produced by the KUL26-610/611 mutant virus (lane 5). For capsids from the mutant virus, no radioactivity was detected at the mobility of the mature scaffold proteins (compare lanes 2, 5, and 6).

Surprisingly, for the cleavage site mutant, little or no radioactivity was detected at the mobility corresponding to VP24 (residues 1 to 247). Protein 21 corresponding to residues 248 to 635 of the UL26 ORF was present in mutant capsids (Fig. 7, compare lanes 2 through 6).

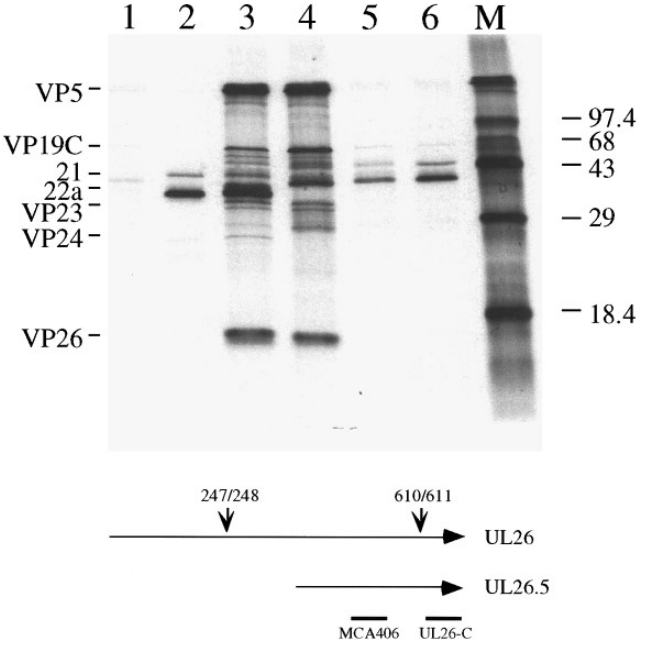

FIG. 7. B capsids formed by KUL26-610/611. Vero cells were infected with KOS (lanes 1 to 3) or with the mutant (lanes 4 to 6) and labeled with [^{35}S]methionine, and nuclear lysates sedimented through sucrose gradients. Material from the single peak of radioactivity for KUL26-610/611 at the fraction corresponding to B capsids for KOS were examined by SDS–PAGE (17% acrylamide) (lanes 3 and 4, respectively). Other samples from the same fractions were precipitated with antibodies specific for the interior portion of the scaffold molecules, antisera MCA406 (lanes 2 and 6), or for the C-terminal 25 amino acids that are normally removed by protease cleavage, antisera UL26-C (lanes 1 and 5). The positions of B capsid proteins and of molecular weight markers (M) are indicated. Shown below the gel is a figure of the two primary translation products of the UL26 ORF, the protease cleavage sites at 247/248 and 610/611, and the recognition region for antibodies MCA406 and UL26-C.

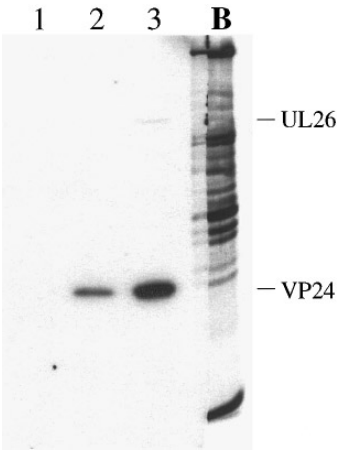

FIG. 8. The synthesis of the UL26 gene product by KUL26-610/611. Vero cells were infected with KOS or the mutant virus and infected cell lysates were prepared and run on 17% gels (SDS–PAGE). Proteins were electroblotted onto nitrocellulose and probed with an antibody specific to amino acids 8 to 14 of UL26. Results for mock-infected cells are shown in lane 1 and for cells infected with KOS (lane 2) and KUL26-610/611 (lane 3). A sample of B capsids from the KOS infections shown in Fig. 7 was included and is designated B.

Western blot experiments were undertaken to confirm that VP24 was synthesized in mutant virus infected cells. Lysates were resolved by SDS–PAGE, along with a wild-type virus sample of radioactively labeled B capsids (Fig. 8). After transfer to nitrocellulose filters the samples were probed with an antibody specific to amino acids 8 to 14 of UL26. A band of radioactivity corresponding to the mobility of VP24 in B capsids was detected for KOS (lane 2)- and mutant virus (lane 3)-infected cells. Cleavage of the UL26 gene product between residues 247 and 248 appears to be normal for the mutant virus. A small amount of radioactivity, probably corresponding to uncleaved translation product of the UL26 transcript, was detected for the mutant virus (lane 3). The primary translation product was not visible for KOS infection, perhaps due to a lower amount of radioactivity present in this sample. We conclude that VP24 was synthesized in KUL26-610/611 infections, but either was not incorporated into capsids or was incorporated and not retained in capsids. Other experiments would be required to determine if cleavage between residues 247 and 248 occurred before or after its incorporation into capsids.

Electron microscope analysis of mutant-infected cells was performed to determine the morphology of the B capsids detected by sedimentation analysis above. Vero cells were infected with KUL26-610/611 and the nuclei in thin sections examined by electron microscopy at 16 h postinfection. Capsids were detected in the nuclei of the majority of the sections examined. The data presented in Fig. 5B show a uniform population of capsids. The capsids show an internal structure consistent with electron scattering due to the presence of scaffold molecules, but not the dark (electron-dense) central cores typical of C capsids.

The first step in capsid growth is an interaction between VP5 and 22a and with the UL26 gene products. Adjacent complexes are stabilized by interaction with VP19C and VP23. If cleavage at the maturation site of 22a and the UL26 gene product occurs prior to VP19C and VP23 interactions, one would not observe capsid formation. Capsid shell precursors were not observed in extracts of cells infected with the VP19C null mutant. Therefore, we wondered if precursors would form if the maturation site of UL26 was also blocked in the VP19C null mutant. To determine if this was the case we isolated a double mutant virus following infection of C32 cells with KΔ19C and KUL26-610/611. The double mutant was identified by growth on C32 cells, but not on cells that expressed either UL26 or UL38 gene products. The double mutant virus was used to infect Vero cells in the presence of [^{35}S]methionine and the nuclei were lysed and sedimented through sucrose gradients, and fractions examined by SDS–PAGE (data not shown). There was no peak of radioactivity corresponding to capsid proteins in any of the fractions. We conclude that the protease activity present in null mutants of capsid proteins does not preclude the formation of capsid precursors.

DISCUSSION

A cell line was isolated that supports the growth of null mutant viruses in all of the essential capsid genes. It was used to isolate a null mutant in the gene encoding VP19C (UL38), a mutant virus blocked at the maturation cleavage site in UL26, and a virus encoding both mutations. The null mutant virus, KΔ19C, did not form detectable precursor structures as judged by sedimentation and electron microscope analyses. We have now constructed null mutant viruses in the three essential gene products that comprise the capsid shell. These viruses encode mutations that abrogate expression of VP5, VP23, or VP19C and do not form detectable capsid precursor structures by the sedimentation procedures used. We conclude that the formation of capsid shells requires the simultaneous presence of all three essential proteins (VP5, VP19C, and VP23). It has also been reported that capsid shells are formed when the components of the capsid shell are present in a virus that encodes null mutants in the protease (VP24) and scaffold (22a and 21) (Desai *et al.,* 1994). Therefore, capsid shells are formed when all of the essential molecules are present and in the absence of the protease and scaffold molecules. However, these capsid shells sediment more rapidly than B capsids, presumably because they form extended sheets that lack the correct curvature of authentic B capsids. Therefore, one of the functions of the scaffold is to provide the correct curvature and/or closure of the shell.

In contrast to the results reported here, 70-nm structures that could correspond to capsid precursors have been observed in insect cells infected with baculoviruses that express UL19 (VP5) and UL38 (VP19C) gene products (Rixon *et al.,* 1996). In addition, Thomsen *et al.* (1994) reported the formation of 50-nm particles when baculoviruses were used that expressed VP5, VP19C, and the scaffold protein, 22a, but the particles contained only VP5 and VP19C. The particles observed in both studies may result from the same molecular interactions that occur between VP5 and VP19C during infection. Infection of nonpermissive mammalian cells with the K23Z mutant, which expresses VP5, VP19C, and the protease and scaffold molecules, did not form these structures, as noted in the last paragraph. The nuclear lysate and sedimentation conditions employed were the same as used by Thomsen *et al.* (1994) and by Rixon *et al.,* (1996). We conclude that the particles formed in the baculovirus system are not formed in infected mammalian cells. Nevertheless, the interactions between VP5 and VP19C used to form the particles in infected insect cells may occur at the same amino acid residues of the two capsid proteins as are found when VP23 is also present.

C32 cells were also used to isolate a mutant virus, KUL26-610/611, that is altered at the maturation cleavage site of UL26 (between amino acids 610 and 611). The mutation was lethal and therefore required a cell line that expressed UL26 for its propagation. The protease in the mutant virus encoded by UL26 is active and cleaved the gene product between the Ala and Ser residues at 247 and 248, to produce VP24 and 21, the N- and C-terminal proteins, respectively. B capsid formation requires the presence of the C-terminal residues of UL26, which are known to interact with VP5, and B capsids are formed by KUL26-610/611 during infection of Vero cells. The scaffold proteins (22a and 21) in the B capsids are uncleaved. C capsids that contain viral DNA are not formed so the block in viral growth is at this stage of morphogenesis. Interestingly, the N-terminal cleavage product of UL26, VP24, normally a component of capsids and virions, is not present or if present it is at a low copy number in the B capsids. If, as is likely, VP24 was initially present in the capsids, its retention is dependent on maturation site cleavage and/or a conformation change that occurs after cleavage.

The simplest model for B capsid formation is that VP5 pentamers and hexamers form (in combination with protease and scaffold molecules), and adjacent VP5 capsomers are held together by VP19C and VP23. The model is based on the recent reports of capsid formation *in vitro* using capsid proteins obtained from expression of individual components from baculovirus-infected insect cells (Newcomb *et al.,* 1996) and on the role of VP19C and VP23 in capsid formation from electron microscope reconstruction experiments (Trus *et al.,* 1996). Capsid growth is by accretion of the essential components on a three-dimensional net. Newcomb *et al.* (1996) were able to trace capsid formation from early spherical structures to icosahedral forms found later during incubation of *in vitro* extracts. It remains to be determined whether the spherical to icosahedral transition occurs during virus infections in mammalian cell culture.

MATERIALS AND METHODS

Cells and viruses

Vero cells and transformed cell lines were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (GIBCO-BRL). Virus stocks were prepared as previously described (Person *et al.,* 1976). C32 was used as the permissive cell line for the propagation of mutant viruses. The viruses, A44 and the temperature-sensitive (ts) mutant in VP19C, A44ts2, were obtained from Dr. Peter Sheldrick, 94802 Villejuif, France (Pertuiset *et al.,* 1989).

Plasmids

The 1.4-kb UL38 ORF which specifies VP19C is encoded within a 3.9-kb *Bam*HI/*Sal*I fragment which was obtained from the *Bam*HI-H fragment of KOS DNA and cloned into the same sites of pUC19 (pKUL38). The UL26 ORF, which specifies the protease (VP24) and scaffold (21 and 22a) proteins, is encoded within a 3.9-kb *Fsp*I fragment which was obtained from a UL26-encoding, 6.5-kb *Not*I plasmid (pKNot1, Desai *et al.,* 1994) and cloned into *Hinc*II-cleaved pUC19 (pKUL26). The UL18 and UL19 ORFs, which specify VP23 and VP5, respectively, are encoded within a 7.2-kb *Kpn*I/*Bgl*II fragment obtained from the *Kpn*I-I fragment and was cloned into *Kpn*I/*Bam*HI sites of pUC19 (pKUL18-19). A plasmid (pKΔUL38) that was deleted for almost the entire UL38 ORF was derived from pKUL38. The deletion, 1.26 kb, extended from a *Nru*I site 15 bp upstream of the UL38 start codon to a *Bsi*WI site 150 bp upstream of the stop codon.

Construction of transformed Vero cell lines

The procedure of DeLuca *et al.* (1985) was followed for transformation of Vero cells. Subconfluent monolayers of Vero cells (2×10^6) in 100-mm petri dishes were cotransfected with pSV2neo (2.0 μg) and plasmids pKUL18-19 (15 μg), pKUL26 (4 μg), and pKUL38 (8 μg) using the calcium phosphate precipitation procedure. At 24 h after transfection the cells were harvested and plated at a density of 4×10^5 cells per 100-mm dish in medium containing 1 mg/ml G418 (GIBCO-BRL). The medium was replenished every 3 days. G418-resistant colonies were harvested using perspex cloning chambers (Bellco Glass) and tested for their ability to support the replication of null mutant viruses in each essential capsid gene.

Marker transfer of null mutations

Subconfluent monolayers of cells (1×10^6) in 60-mm dishes were cotransfected with 1 μg linearized plasmid and approximately 5 μg of KOS genomic DNA extracted from crude virion preparations as described previously (Desai *et al.,* 1993). Approximately 48 h after transfection the cell monolayers were harvested, freeze/thawed once, and sonicated and total virus progeny was titered.

Southern blot hybridization

DNA sequences were detected after agarose gel electrophoresis by Southern blot analysis, using random-primer-labeled probes (Boehringer-Mannheim), labeled with [α-^{32}P]deoxycytidine 5′-triphosphate (DuPont-NEN). Fragment sizes in Southern blot experiments were determined from the mobilities of DNA markers that were measured prior to blotting of fragments.

Radiolabeling, immunoprecipitation, and SDS–PAGE

Radiolabeling of KΔ19C-infected cells was carried out using preformed monolayers (1×10^6 cells) in six-well trays (35-mm-diameter well). Infected cell monolayers were rinsed twice with tricine-buffered saline (TBS) (Person *et al.,* 1976) and overlaid with methionine-free Dulbecco's modified Eagle's medium (GIBCO-BRL) containing 100 μCi of [^{35}S]methionine (DuPont-NEN). The cells were lysed in 1.0 ml RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Na deoxycholate, 1% SDS, 1% Triton X-100) plus 0.5 mM tosyllysylchloromethyl ketone (TLCK). Lysates were clarified by centrifugation. For immunoprecipitation of proteins, 250 μl of the lysate was combined with 3 μl of antibody and incubated overnight at 4°C. The next day 50 μl of protein A–Sepharose beads (Sigma) swollen in RIPA buffer was added to the tube and incubated for 2 h at 4°C with continuous rotation. The beads were washed five times with RIPA buffer and resuspended in 25 μl 2× Laemmli sample buffer. Samples (10 μl) were boiled prior to electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses were performed using the mini-protean gel system (Bio-Rad). Gels were cast according to the protocol supplied by the manufacturer, except that *N,N′*-diallytartardiamide (DATD) was the cross-linking agent. Gels were treated to ENhance (DuPont-NEN) and dried prior to autoradiography.

Western blots

Confluent monolayers of cells (1×10^6 cells) were infected with virus at a multiplicity of infection (m.o.i.) of 10 PFU/cell and harvested 24 h after infection. Proteins were separated on 12% acrylamide SDS–PAGE and transferred to NYTRAN (Schleicher & Schuell) according to procedures supplied by the manufacturer (Bio-Rad). Filters were blocked with 1% BLOTTO in TN buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% sodium azide) and exposed to VP19C antibody (NC2) for 90 min, washed extensively, exposed to [^{125}I]-labeled protein A (DuPont-NEN) for 60 min, washed, and air dried prior to autoradiography. Antibodies and washes were in TN buffer. Antibodies prepared against VP19C were provided by Drs. R. Eisenberg and G. Cohen (University of Pennsylvania). Peptide antibodies specific to amino acids 8 to 14 of UL26 (antibody 37-94) were generously provided by Dr. R. LaFemina (Merck Research Laboratories). Peptide antisera specific to residues 611–635 of UL26 (UL26-C) were prepared in rabbits by Research Genetics Inc.

Sedimentation analysis

Confluent monolayers of Vero cells in two 100-mm diameter petri dishes (approximately 1×10^7 cells) were infected at an m.o.i. of 10 PFU/cell. After adsorption, 4 ml of labeling medium was added to each culture. Labeling medium consists of 70% Dulbecco's MEM medium without methionine, 20% F12 (contains 5 μg/ml of methionine and 10% fetal bovine serum (GIBCO-BRL)). Eight hours after infection, 200 μCi of [^{35}S]methionine (DuPont-NEN) was added to each culture and incubation continued until 24 h postinfection. Cells were scraped into the growth medium, and cultures from duplicate plates were combined and pelleted at 3500 *g* for 5 min at 4°C. Pellets were resuspended in TBS, pelleted again, resuspended in 0.5× capsid lysis buffer (500 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 1% Triton X-100, and 0.5 mM TLCK), and left on ice for 5 min and the nuclei pelleted by centrifugation at 3500 *g* for 15 min at 4°C. Nuclei were lysed in 2× capsid lysis buffer and sonicated and the lysate was layered onto a 20 to 50% sucrose gradient (w/v in 300 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, and 0.5 mM TLCK) and centrifuged at 24,000 rpm for 120 min in a Beckman SW41 rotor at 4°C. Fractions were collected and radioactivity determined by liquid scintillation counting. For SDS–PAGE analysis fractions were precipitated with equal volumes of 10% trichloroacetic acid.

Oligonucleotide-directed *in vitro* mutagenesis

Mutagenesis employed the Muta-Gene phagemid kit supplied by Bio-Rad. The *Kpn*I-T fragment, encoding UL26, was cloned into the same site in the pTZ19U vector which includes the single-strand origin of replication of the bacteriophage f1. The plasmid was transfected into a uracil repair-deficient bacterium (CJ236) and superinfected with helper phage R408 (Russel *et al.,* 1986) to yield a large proportion of phage whose single-stranded DNA contains uracil residues at a limited number of thymine sites. This DNA was used as template during the oligonucleotide-directed *in vitro* mutagenesis reaction. Following synthesis the double-stranded DNA was transformed into the uracil repair-proficient strain (DH5α)

which results in inactivation of uracil containing DNA in the parental stand.

Electron microscopy

Confluent monolayers of infected cells were fixed in 4% formaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The samples were then postfixed in 1% osmium tetroxide, stained with uranyl acetate, dehydrated through a graded series of ethanols, and embedded in Polybed 812 resin. Thin sections were cut at 0.1 μm in a Porter Blum MT-1 ultramicrotome, mounted on carbon-coated grids, stained with lead citrate and uranyl acetate, and examined at 60 kV under a JOEL 1200EX electron microscope.

Data preparation

Autoradiographs were scanned on a Umax Powerlook II scanner. The images were scanned at 300 dots per inch into Adobe Photoshop 3.0 and were transported as PICT files into Microsoft Powerpoint for figure presentation and printing.

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